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Influence of temperature on the enzymic semisynthesis of human insulin by coupling and transpeptidation methods

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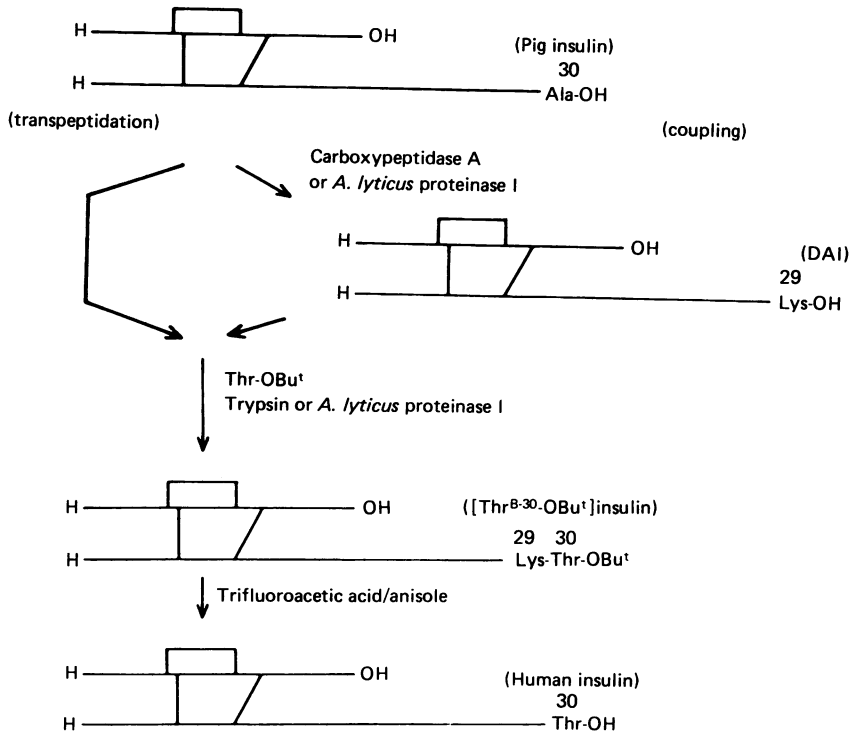
The influence of temperature of enzymic semisynthesis of human insulin ester was determined by using coupling and transpeptidation methods with trypsin and *Achromobacter lyticus* proteinase I as catalysts. The optimal reaction conditions were studied at the selected temperatures of 25, 12 and 4 °C. The results showed that the synthesis rates by both methods with trypsin increased as the temperature increased, but the final product yield correspondingly decreased. Therefore the reaction with trypsin should be done below 12 °C, preferably at 4 °C. This agrees well with the stability of trypsin at these temperatures. When the catalyst was *Achromobacter lyticus* proteinase I, no such complex temperature effects were observed, and the findings indicated that the reactions should be conducted below 37 °C for enzyme stability.

INTRODUCTION

Pig insulin can be enzymically converted into human insulin derivatives (refer to Scheme 1): (1) by preparing des-B-30-alanine-(pig insulin) (DAI) by digesting pig insulin with carboxypeptidase A (Schmitt & Gattner, 1978) or *Achromobacter lyticus* proteinase I (EC 3.4.21.50) (Masaki *et al.*, 1978) and then coupling it with Thr-X (X = blocking residues) (two-step reaction), or (2) by subjecting Ala-B-30 in pig insulin to transpeptidation with Thr-X (one-step reaction). The former is the 'coupling' method and the latter the 'transpeptidation' method. [From the results of their m.s. investigation, Rose *et al.* (1984) concluded that the one-step reaction is not mechanistically a transpeptidation; nevertheless, for convenience, we shall refer to it as such in this paper.] The experimental conditions for coupling with trypsin and *A. lyticus* proteinase I have been studied by us



Scheme 1. Enzymic conversion of pig insulin into human insulin

Abbreviations: DAI, des-B-30-alanine-(pig insulin); Bz-, benzoyl; -OEt, ethyl ester; -OBu^t, t-butyl ester; -Nan, *p*-nitroanilide; abbreviated designations of amino acids, peptides or their derivatives conform to the tentative rules of the IUPAC–IUB Commission on Biochemical Nomenclature; except when specified, the constituent amino acids were all of the L-configuration.

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(Morihara *et al.*, 1979, 1980), and those for transpeptidation by trypsin have been done by Markussen (1981), Jonczyk & Gattner (1981) and Rose *et al.* (1983), that by carboxypeptidase Y by Breddam *et al.* (1981), and by *A. lyticus* proteinase I by Morihara & Oka (1983).

One of the merits of enzymic peptide-bond synthesis is that the reaction can be performed under mild conditions. However, carefully controlled conditions are required for syntheses in which the reaction mixtures contain a high concentration of organic co-solvents, such as seen in semisynthesis of human insulin (Morihara *et al.*, 1979, 1980; Markussen, 1981; Jonczyk & Gattner, 1981; Morihara & Oka, 1983; Rose *et al.*, 1983). Sakina *et al.* (1986) also indicated that the semisynthesis of [B-30-leucine]insulin by the coupling method differed depending on the temperatures used; the yield with trypsin was considerably lower at 37 °C than that at 25 °C. This was assumed to be due to the instability of the enzyme at 37 °C. Markussen (1981) performed the transpeptidation reaction at 12 or 4 °C with trypsin as a catalyst. Yagisawa (1981) reported the loss of synthetic activity of trypsin in 50% (v/v) dioxan at pH 4.37 and temperatures above 15 °C. The present systematic study also examined the influence of the reaction temperature on the semisynthesis of human insulin derivatives by both coupling and transpeptidation methods with trypsin and *A. lyticus* proteinase I.

EXPERIMENTAL

Materials

Pig insulin (25 units/mg) was obtained from Calbiochem–Behring, Los Angeles, CA, U.S.A. *A. lyticus* proteinase I and Bz-Lys-Nan were supplied by Wako Pure Chemicals, Osaka, Japan. Trypsin (type XIII, tosylphenylalanylchloromethane-treated) and carboxypeptidase A (di-isopropyl phosphorofluoridate-treated) were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Bz-Arg-OEt was obtained from the Protein Research Foundation, Minoh, Osaka, Japan. Zn-free insulin was prepared by gel filtration (Sephadex G-50) with 1 M-acetic acid as eluent, followed by freeze-drying. DAI was obtained by digestion of pig insulin with carboxypeptidase A by a modification of the method of Schmitt & Gattner (1978). The DAI content of the preparation was determined to be more than 90% from the amino acid analysis. Thr-OBu^t was prepared according to the usual chemical method (Wünsch *et al.*, 1965; Naithani & Föhles, 1978).

Methods

Except where otherwise specified, the coupling reaction was carried out in a reaction mixture (0.17 ml) containing 10 mM-DAI, 0.8 M-Thr-OBu^t (oil), 50% dimethylformamide/ethanol (1:1, v/v) and 26% water, adjusted to pH 7.0 with acetic acid. The total volume was adjusted to 100% by addition of Thr-OBu^t and acetic acid. Throughout this work pH was measured with an uncorrected glass electrode (Horiba 6029-10T). The transpeptidation reaction was usually performed in a reaction mixture (0.17 ml) containing 10 mM Zn-free pig insulin, 0.8 M-Thr-OBu^t, 45–60% dimethylformamide/ethanol and 16–31% water with the pH adjusted to 6.0 (for trypsin) or 6.5 (for *A. lyticus* proteinase I). The reaction was performed at 25, 12 and 4 °C.

The semisynthesis of human insulin ester was determined quantitatively by using h.p.l.c. with a column of Nucleosil $5C_{18}$ and elution with a linear gradient of acetonitrile, as shown in Fig. 1. The yield of the synthesis was calculated from the peak areas as the ratio of the peak area corresponding to human insulin ester to the total peak area of insulin and its products derived from the starting DAI or pig insulin. This method is better than the previous one (Morihara *et al.*, 1979, 1980), since the latter method might have missed the amounts of byproducts derived from DAI or insulin for the determination.

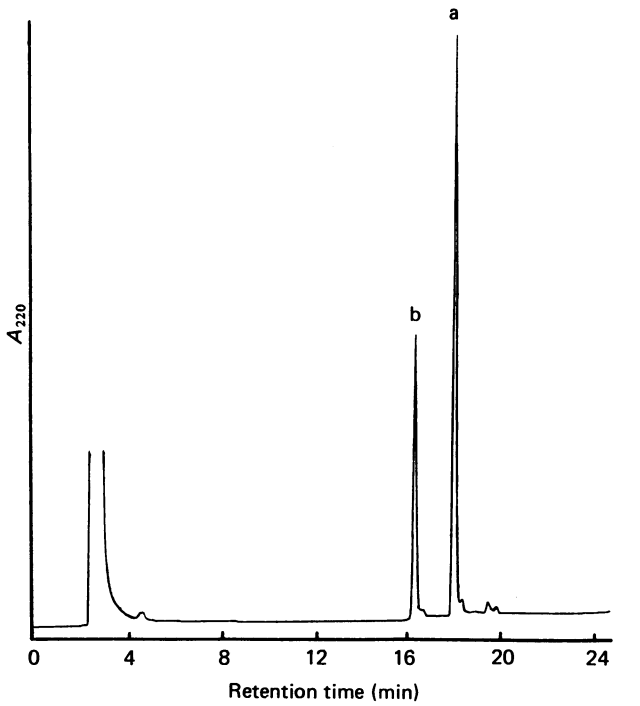

Fig. 1. H.p.l.c. profile of human insulin ester (a) and starting insulin or DAI (b)

A 0.4 cm × 23 cm column of Nucleosil $5C_{18}$ was used, with as eluent 5 mM-phosphate buffer, pH 3.0, containing 5 mM-sodium n-butanesulphonate and 50 mM-Na_2SO_4, with a 27–48% linear gradient of acetonitrile in 21 min. The flow rate was 1 ml/min. Detection was by the absorbance at 220 nm.

The enzyme activity of trypsin was determined at 25 °C and pH 7.6 with Bz-Arg-OEt as substrate, in which a change in absorbance at 253 nm was recorded in accordance with the method described previously (U.S. Pharmacopeia National Formulary, 1980). The activity of *A. lyticus* proteinase I was determined at 30 °C and pH 9.5 with Bz-Lys-Nan as substrate by using the method described by Masaki *et al.* (1981), where the release of *p*-nitroaniline was monitored at 405 nm.

RESULTS

Optimal conditions for semisynthesis of human insulin ester at various temperatures

Effect of pH. To determine the optimum pH for semisynthesis at various temperatures, the reaction mixtures of various pH values were kept at 25, 12 and 4 °C. The results are summarized in Fig. 2. For the

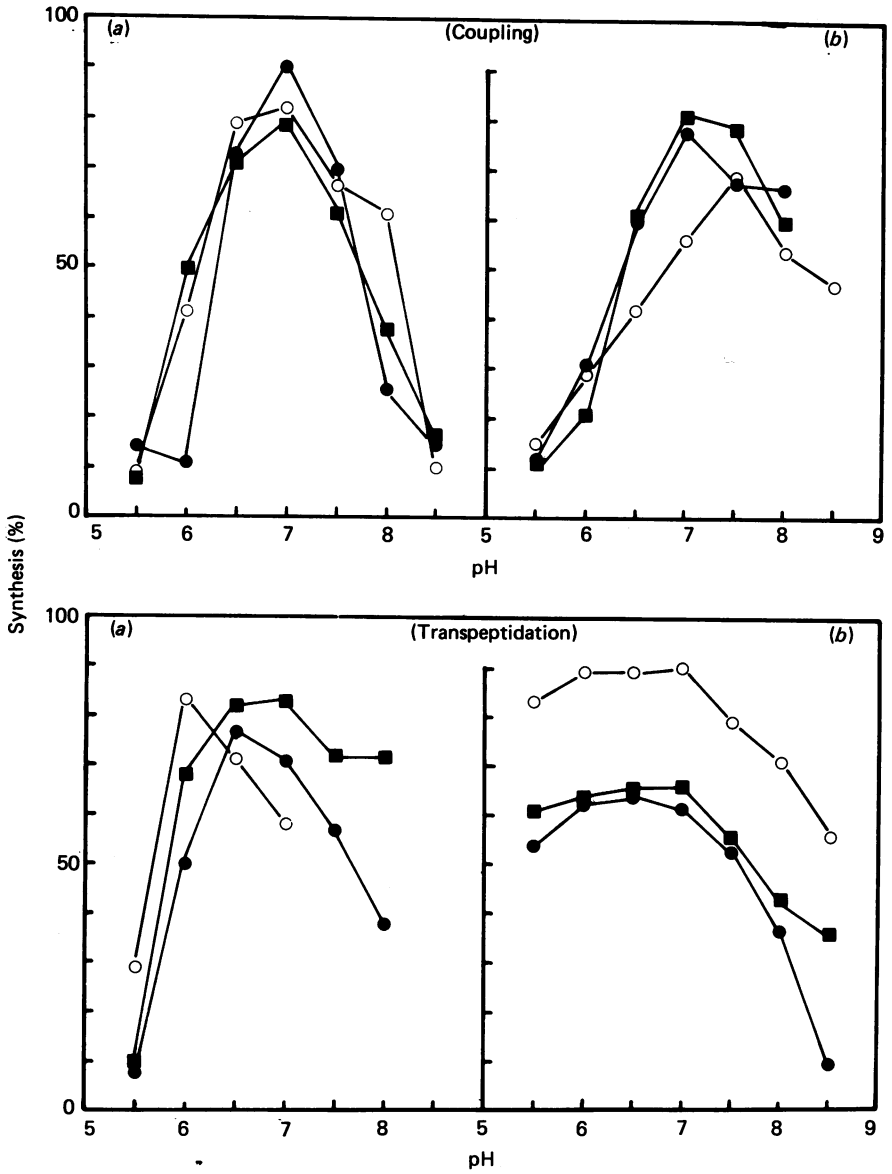

Fig. 2. Effect of pH on semisynthesis of human insulin ester with trypsin (*a*) and *A. lyticus* proteinase I (*b*) at 25 °C (●), 12 °C (○) or 4 °C (■)

Except where otherwise specified, the reaction mixture contained 10 mM-DAI (coupling) or -insulin (transpeptidation), 0.8 M-Thr-OBu^t, 50% dimethylformamide/ethanol mixture and 26% water, adjusted to various pH values with acetic acid. A 45% or 60% dimethylformamide/ethanol mixture (31% or 16% water, correspondingly) was involved in the reaction mixture for coupling with *A. lyticus* proteinase I or transpeptidation with *A. lyticus* proteinase I respectively. The enzyme concentration and reaction time were as follows. Coupling (*a*) ● (10 μM, 2 h), ○ (1 μM, 24 h), ■ (2 μM, 24 h); (*b*), ● (2 μM, 6 h), ○ (5 μM, 2 h), ■ (5 μM, 24 h). Transpeptidation: (*a*) ● (333 μM, 6 h), ○ (333 μM, 24 h), ■ (333 μM, 24 h); (*b*) ● (100 μM, 2 h), ○ (100 μM, 24 h), ■ (100 μM, 24 h).

coupling reaction, the optimum pH for trypsin and *A. lyticus* proteinase I was near 7.0 at all the temperatures tested except in the case of *A. lyticus* proteinase I at 12 °C, for which it was pH 7.5. For the transpeptidation reaction, the neutral pH was also optimal for both enzymes at all temperatures tested except in the case of trypsin at 12 °C, for which it was pH 6.0. We also found that the optimum pH of *A. lyticus* proteinase I was broad (6–7) at all the temperatures tested.

Morihara & Oka (1983) have previously reported that the optimum pH of the transpeptidation reaction with *A. lyticus* proteinase I was at about 5. Rose *et al.* (1983) found that the optimum pH for the transpeptidation reaction with trypsin was at 5.3–5.6. The difference in the results might be ascribed to the use of an uncorrected glass electrode to check the pH adjustment. Another reason might be the difference in methods used for quantitative determination of product yield. The present method is better for determining the absolute amount of the product than that used in the previous studies (Morihara *et al.*, 1979, 1980), as described in the Experimental section of the present paper.

Effect of organic co-solvent. To determine the effects of the kinds of organic co-solvents used in the semisynthesis, the experiments were first performed by using the coupling method at 12 °C. The results summarized in Fig. 3 indicate that dimethylformamide/ethanol and dimethyl sulphoxide/ethanol mixtures were useful for the trypsin reaction, followed by the less efficient ethanol, with dimethylformamide, butane-1,4-diol and dimethyl sulphoxide being inefficient. On the other hand, most of the organic co-solvents used were suitable for the synthesis with *A. lyticus* proteinase I. Thus the following experiment was performed in reaction mixtures containing dimethylformamide/ethanol (1:1, v/v).

The effects of the concentration of dimethylformamide/ ethanol on semisynthesis by coupling and transpeptidation with trypsin and *A. lyticus* proteinase I at various temperatures were determined as shown in Fig. 4. In the coupling method, the optimum concentration for trypsin was 50% (water content 26%) at all the temperatures tested, whereas that for *A. lyticus* proteinase I was 60% (water content 16%) at 12 °C, 50% at 25 °C, and 45% (water content 31%) at 4 °C. In the last case, a reaction time longer than 24 h might be necessary because of the low reaction rate. In the transpeptidation reaction, the optimum concentration for trypsin was 45% at 25 °C, 50% at 12 °C and 55% (water content 21%) at 4 °C, and that for *A. lyticus* proteinase I was 60% at all the temperatures tested.

On the basis of these findings, the optimal conditions were selected for the coupling or transpeptidation reaction with trypsin or *A. lyticus* proteinase I as a catalyst at 25, 12 and 4 °C. The following experiments were conducted under the chosen conditions.

Influence of temperatures on semisynthesis of human insulin ester

Coupling. Reaction mixtures containing various amounts of enzyme were kept at 25, 12 and 4 °C at the respective optimal conditions. At various intervals, a small amount of the reaction mixture was withdrawn to determine the extent of synthesis. The results summarized in Figs. 5(*a*) and 5(*b*) indicate that the initial rate of synthesis in the coupling reaction with either enzyme increases as the temperature increases (25 °C > 12 °C > 4 °C), but the final product yield of a 24 h reaction appears to decrease as the temperature increases: 82% yield with 2 μM-trypsin and 85% yield with 5 μM *A. lyticus* proteinase I at 25 °C, 85% yield with 5 μM-trypsin and 5 μM *A. lyticus* proteinase I at 12 °C, and 90% yield with 2 μM-trypsin and 5 μM *A. lyticus* proteinase I at 4 °C. Figs. 5(*a*) and 5(*b*) also indicate that the rate of synthesis with trypsin is higher (more than 2-fold) than that with *A. lyticus* proteinase I. [In a preliminary note (Morihara & Oka, 1983) it was reported that the coupling activity of *A. lyticus* proteinase I was about 10-fold higher than that of trypsin. The discrepancy with the present result might be ascribed to a smaller quantity of active trypsin in the preparation used for the previous study.]

Transpeptidation. Similar experiments were undertaken with the transpeptidation reaction at 25, 12 and 4 °C under the respective optimal conditions. Note from the results summarized in Figs. 6(*a*) and 6(*b*) that a considerably large amount of enzyme was required for the transpeptidation reaction (more than 100-fold) than the coupling one. This tendency was more marked with trypsin than with *A. lyticus* proteinase I. This amount of enzyme for transpeptidation with trypsin is comparable with those described by other investigators (insulin/trypsin = 10:1, by weight; Jonczyk & Gattner, 1981; Markussen, 1981; Rose *et al.*, 1983). As seen with the coupling reaction, Figs. 6(*a*) and 6(*b*) indicate that the initial rate of synthesis with either trypsin or *A. lyticus* proteinase I becomes faster as the temperature increases, but the final product yield of trypsin from the 24 h reaction appears to decrease as the temperature increases: 69% yield with 500 μM enzyme at 25 °C, 82% yield with 500 μM enzyme at 12 °C, and 80% yield with 500 μM enzyme at 4 °C. As for the reaction with *A. lyticus* proteinase I, the final product yield did not depend on temperature over the range tested: about 90% yield with 100 μM enzyme at 25, 12 and 4 °C (48 h reaction). Figs. 6(*a*) and 6(*b*) also indicate that the synthetic activity of

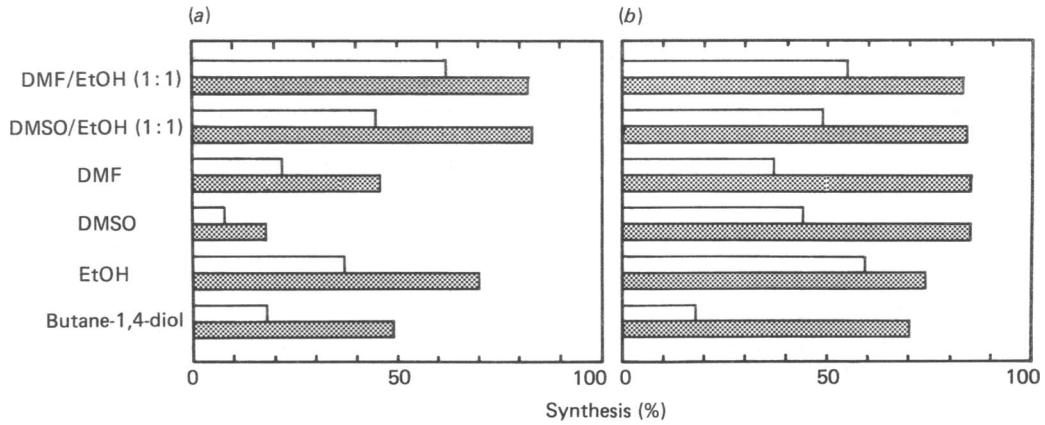

Fig. 3. Effect of the kinds of organic co-solvents on semisynthesis of human insulin ester by the coupling method with trypsin (*a*) and *A. lyticus* proteinase I (*b*) at pH 7.0 and 12 °C

The reaction mixture contained 10 mM-DAI, 0.8 M-Thr-OBu^t, 50% organic co-solvent and 26% water, adjusted the pH to 7.0 with acetic acid. Various kinds of organic co-solvents are shown in the Figure (abbreviations: DMF, dimethylformamide; DMSO, dimethyl sulphoxide). The enzyme concentration and reaction time were as follows: (*a*) □ (1 μM, 6 h), ▩ (1 μM, 24 h); (*b*) □ (5 μM, 2 h), ▩ (5 μM, 24 h).

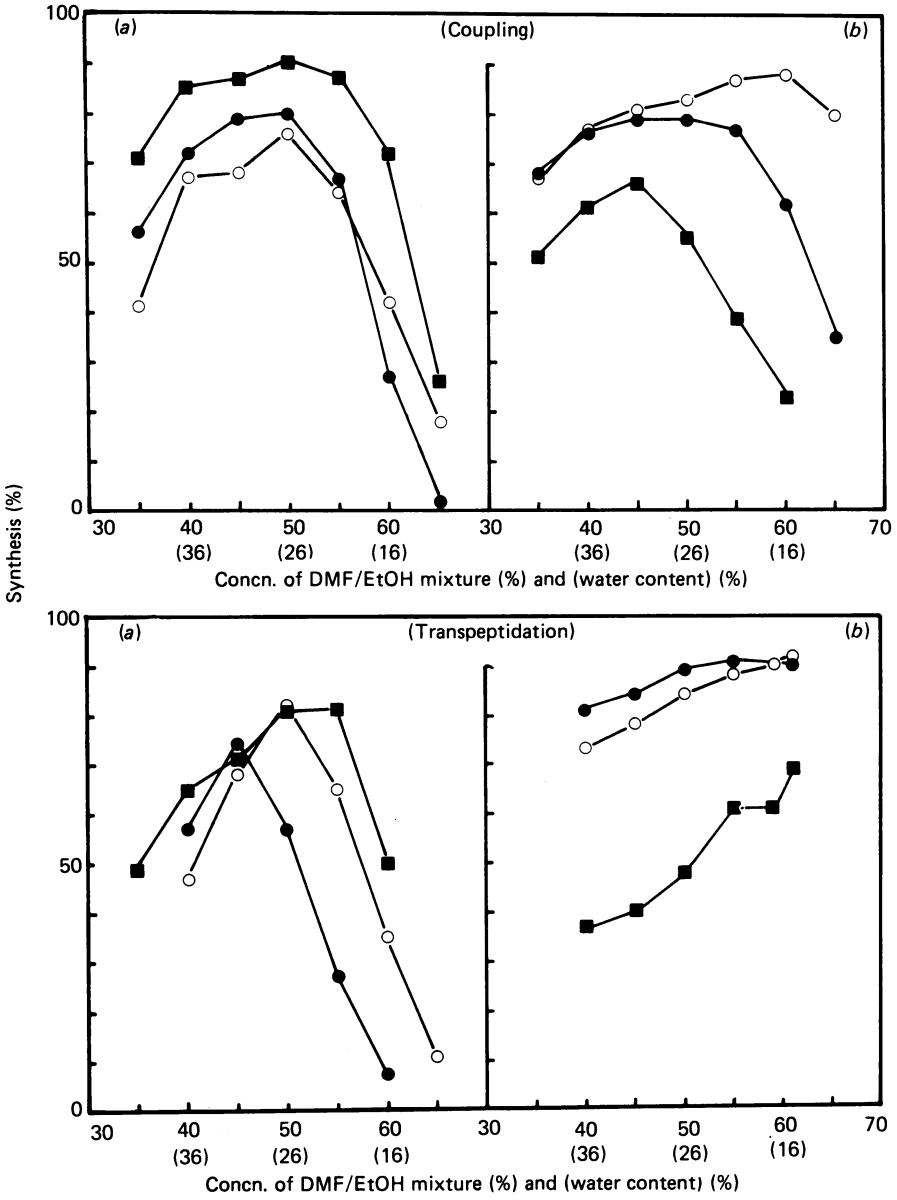

Fig. 4. Effect of the concentration of dimethylformamide/ethanol mixture on semisynthesis of human insulin ester with trypsin (*a*) and *A. lyticus* proteinase I (*b*) at 25 °C (●), 12 °C (○) and 4 °C (■)

The reaction mixture contained 10 mM-DAI (coupling) or- insulin (transpeptidation), 0.8 M-Thr-OBu^t and various concentrations of dimethylformamide/ethanol (DMF/EtOH) mixture (1:1, v/v) and water, as shown in the Figure. The pH was adjusted to 7.0. The enzyme concentration and reaction time were as follows. Coupling (*a*) ● and ○ (1 μM, 6 h), ■ (1 μM, 24 h); (*b*) ● (1 μM, 7 h), ○ (5 μM, 24 h), ■ (1 μM, 24 h). Transpeptidation: (*a*) ● (333 μM, 2 h), ○ and ■ (333 μM, 24 h); (*b*) ●, ○ and ■ (100 μM, 24 h).

A. lyticus proteinase I was higher than that of trypsin (more than 2-fold at 12 °C) to reach the same degree of final product yield by 24 h reaction. The relative synthetic activity of the two enzymes is better measured by the initial reaction rate, and the initial reaction rates show *A. lyticus* proteinase I to be the more active enzyme in transpeptidation, differing from the result of coupling as shown in Figs. 5(*a*) and 5(*b*).

Enzyme stability in the presence of high concentration of organic co-solvent

Coupling or transpeptidation reaction was performed in the presence of a high concentration of organic co-solvents, which might have inactivated some of the enzyme activity of the reaction mixture during synthesis. The stabilities of trypsin and *A. lyticus* proteinase I were determined in solution containing 65% dimethylformamide/ethanol (water content 35%). As shown in Fig. 7, trypsin was markedly more sensitive to the increase of temperature in comparison with *A. lyticus* proteinase I. Trypsin became inactive at 37 °C within 2 h even in the presence of Ca^{2+}. Inactivation also occurred at 25 °C, but was not as marked as at 37 °C and was slowed by the presence of Ca^{2+}. Below 12 °C typsin was almost completely stable for 24 h whether in the presence or absence of Ca^{2+}. On the other hand, *A. lyticus* proteinase I was almost completely stable below 37 °C for 24 h.

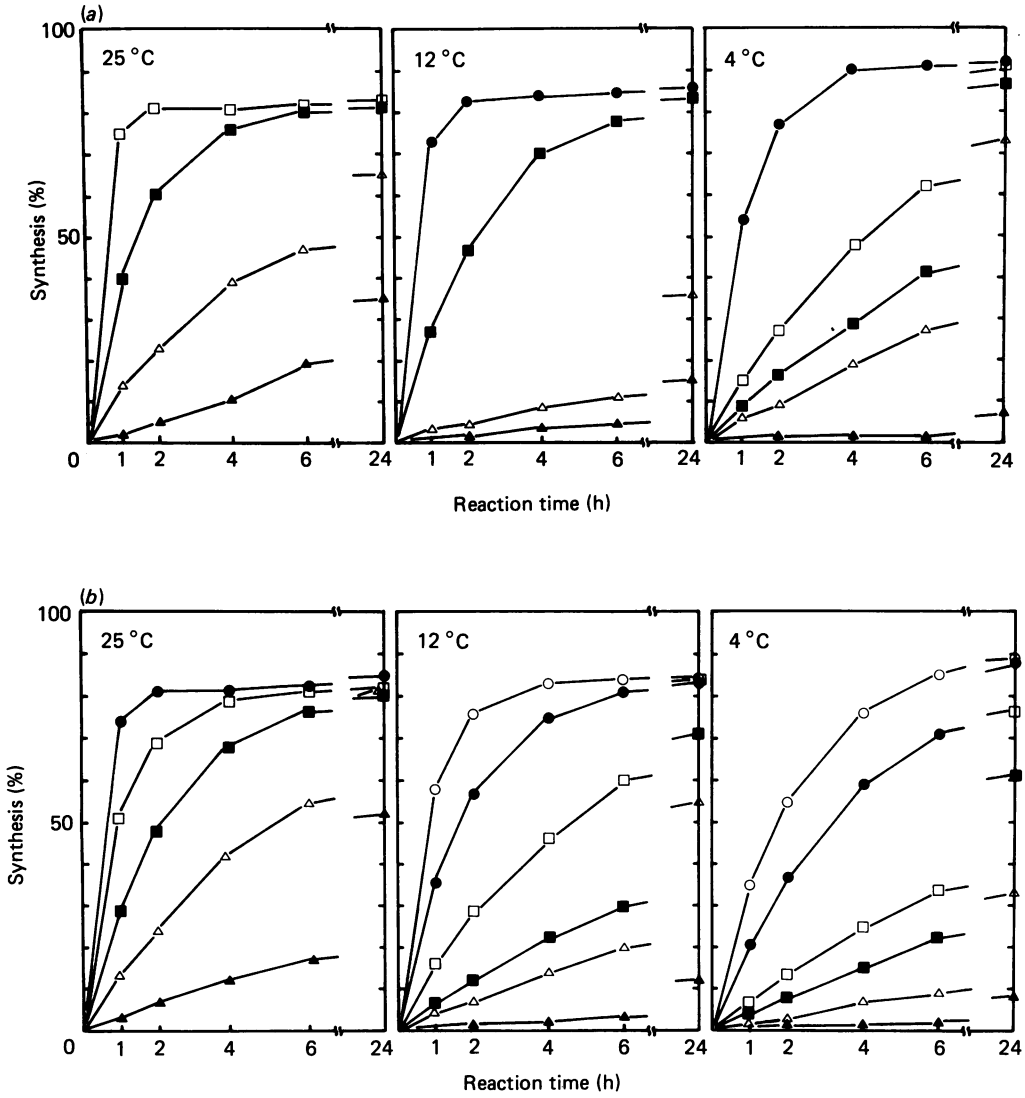

Fig. 5. Effect of enzyme concentration on semisynthesis of human insulin ester by the coupling method with (*a*) trypsin and (*b*) *A. lyticus* proteinase I at various temperatures

(*a*) The reaction mixture contained 10 mM-DAI, 0.8 M-Thr-OBu^t, 50% dimethylformamide/ethanol mixture and 26% water, pH adjusted to 7.0. The enzyme concentrations were as follows: 5 μM (●), 2 μM (□), 1 μM (■), 0.5 μM (△) and 0.1 μM (▲). (*b*) The reaction mixture contained 10 mM-DAI, 0.8 M-Thr-OBu^t, 50% dimethylformamide/ethanol mixture and 26% water for the reaction at 25 °C, 60% dimethylformamide/ethanol mixture and 16% water for at 12 °C, or 45% dimethylformamide/ethanol mixture and 31% water at 4 °C. The pH was adjusted to 7.0. The enzyme concentrations were as follows: 10 μM (○), 5 μM (●), 2 μM (□), 1 μM (■), 0.5 μM (△) and 0.1 μM (▲).

DISCUSSION

The optimal conditions for semisynthesis of human insulin ester by either coupling or transpeptidation method were determined at 25, 12 and 4 °C with trypsin and *A. lyticus* proteinase I as catalysts. The reaction was done in the presence of high concentrations of an amine component and an organic co-solvent at about neutral pH irrespective of the difference in reaction types or enzyme sources at all the temperatures tested, although small differences were noted. We found that expected side reaction such as cleavage of the Arg$^{B\text{-}22}$–Gly$^{B\text{-}23}$ bond was negligible in the reaction with trypsin under the optimal conditions of both the coupling and transpeptidation methods. This was true also with *A. lyticus* proteinase I, which had been expected because of its narrow specificity against only L-lysine.

Trypsin and *A. lyticus* proteinase I were compared as to the amount of enzyme required to reach the maximum product yield after 24 or 48 h reaction. The initial velocity of synthesis with both enzymes increased with increase of temperature, but the final product yield did not always correspond to this, especially with trypsin. The maximum product yield by 24 h reaction (the yield being shown in the parentheses) was obtained in the coupling method (90%) with 2 μM-trypsin at 4 °C and in the transpeptidation one (82%) with 200 μM-trypsin at 12 °C. With *A. lyticus* proteinase I, the maximum product yield was obtained at 4 °C with 2 μM enzyme for the coupling (90%) and at 25 °C with 50 μM enzyme for

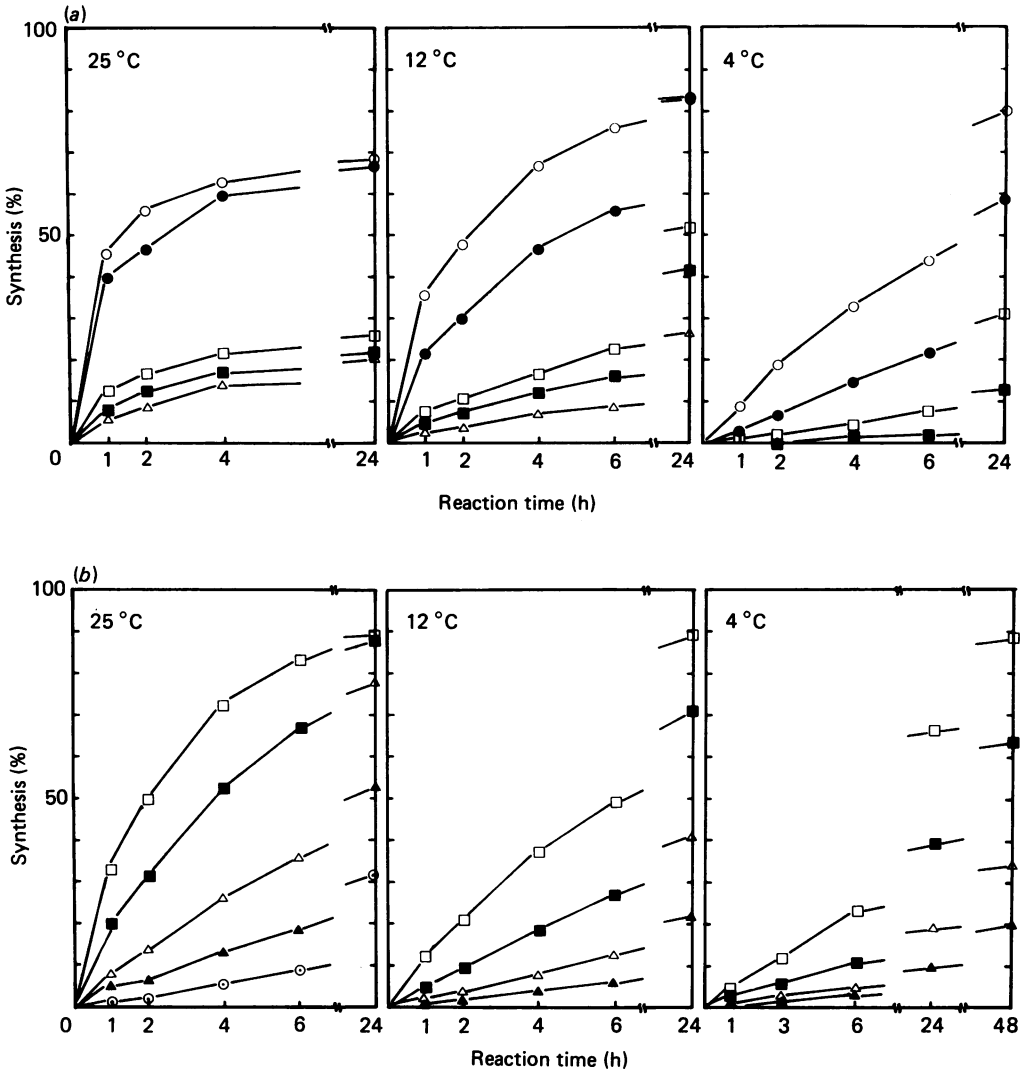

Fig. 6. Effect of enzyme concentration on semisynthesis of human insulin ester by the transpeptidation method with (*a*) trypsin and (*b*) *A. lyticus* proteinase I at various temperatures

(*a*) The reaction mixture contained 10 mM-insulin, 0.8 M-Thr-OBu^t, and 45% dimethylformamide/ethanol mixture and 31% water for the reaction at 25 °C, or 50% dimethylformamide/ethanol mixture and 26% water for those at 12 and 4 °C. The pH was adjusted to 6.5 for the reactions at 25 and 4 °C, and to 6.0 for that at 12 °C. The enzyme concentrations were 500 μM (○), 200 μM (●), 100 μM (□), 50 μM (■) and 20 μM (△). (*b*) The reaction mixture contained 10 mM insulin, 0.8 M-Thr-OBu^t, 60% dimethylformamide/ethanol mixture and 16% water, pH adjusted to 6.5. The enzyme concentrations were 100 μM (□), 50 μM (■), 20 μM (△), 10 μM (▲) and 5 μM (⊙).

transpeptidation (90%). Therefore the amount of trypsin required for the maximum synthesis by the coupling method was comparable with that of *A. lyticus* proteinase I, but that of trypsin by the transpeptidation method was 4–5 times that of *A. lyticus* proteinase I. However, because the price of crystalline trypsin is one-threehundredth the cost of *A. lyticus* proteinase I, trypsin is economically superior to *A. lyticus* proteinase I for the synthesis.

A stability test in the presence of a high concentration of organic co-solvent indicated that trypsin became instable above 25 °C but was stable below 12 °C, whereas *A. lyticus* proteinase I was stable below 37 °C. Therefore the semisynthesis of human insulin ester with trypsin should be done below 12 °C notwithstanding the differences in methods. Markussen (1981) performed the transpeptidation reaction at 12 or 4 °C. As for the coupling with *A. lyticus* proteinase I, the final product yield was also high (90%) at 4 °C in comparison with those at 25 and 12 °C.

This brings us to the question of which enzymic method is suitable for industrial production of human insulin: coupling or transpeptidation. In the latter, pig insulin can be used directly as the starting material, although the amount of enzyme required for reaction with trypsin is more than 100 times that for the coupling method. Although the factor of 25 applied for *A. lyticus* proteinase I, the latter is rejected for industrial purposes on the basis of cost. On the other hand, DAI can easily be prepared by either carboxypeptidase A or *A. lyticus* proteinase I in more than 90% yield. Therefore both the quantitative and qualitative factors in the synthesis must be considered.

Immobilized enzymes are now being used for industrial

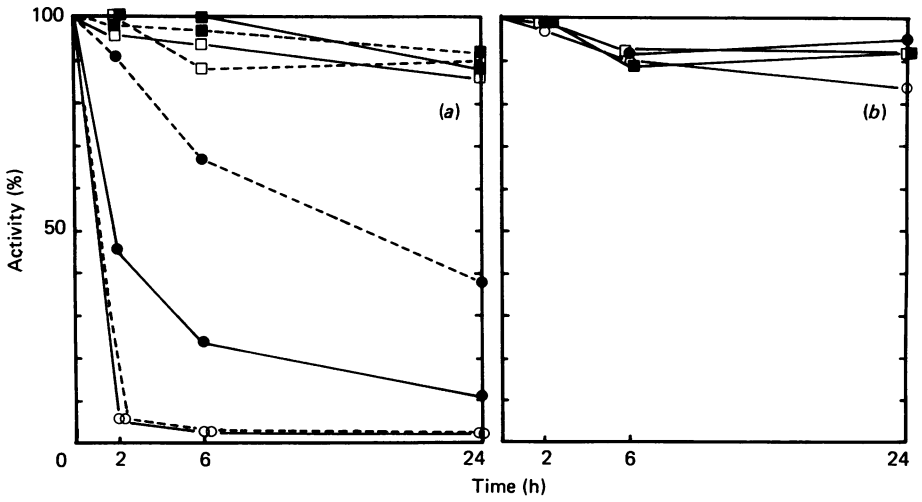

Fig. 7. Effect of high concentration of organic co-solvent on the stabilities of trypsin (*a*) and *A. lyticus* proteinase I (*b*) at various temperatures

The reaction mixture contained 5 μM enzyme, 65% dimethylformamide/ethanol mixture and 35% water, pH adjusted to 7.0, which was then kept at 37 °C (○), 25 °C (●), 12 °C (□) or 4 °C (■) in the presence (----) or absence (——) of 2 mM-$CaCl_2$. At various intervals the remaining enzyme activity was determined by the method described in the Experimental section.

production because enzyme loss is small over long periods of continuous production. Such methods are more economical than conventional methods with soluble enzymes. Nilson & Mosbach (1984) have shown that immobilized chymotrypsin can be used in 90% butane-1,4-diol to effect peptide synthesis, and yield of peptide and stability of the immobilized enzyme were increased at lower temperatures. Muneyuki *et al.* (1982) and Oka *et al.* (1983) have reported that immobilized *A. lyticus* proteinase I attached to silica gel/polyglutamic acid could be used for semisynthesis of human insulin ester by coupling but not by transpeptidation. The large amount of enzyme that had to be attached to the support could not be produced for the transpeptidation method. Recently we found (K. Morihara, Y. Ueno & K. Sakina, unpublished work) that immobilized trypsin can also be used for the coupling method at 4 °C but not for the transpeptidation one. Rose *et al.* (1983), however, have reported using immobilized trypsin attached to beaded Sepharose for the transpeptidation reaction.

We conclude that immobilized trypsin should be most useful for industrial production of human insulin, with the temperature kept below 12 °C and preferably at 4 °C. The transpeptidation method is not as efficient as the coupling method because it requires much enzyme, resulting in difficulty in the use of the immobilized enzyme.

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